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Expression Profiles of Tyrosine Kinases in Cultured Follicular Papilla Cells *Versus* Dermal Fibroblasts

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Tyrosine kinases play crucial roles in cell differentiation and proliferation. Using degenerative primed PCR followed by differential display, we analyzed the tyrosine kinase expression profiles of cultured rat follicular papilla (FP) cells *versus* dermal fibroblasts. We showed that c-met, cdc2, and tec were preferentially expressed in cultured FP cells, whereas α-platelet-derived growth factor receptor (α-PDGFR) was preferentially expressed in cultured fibroblasts. The cell type specificity of these tyrosine kinases was confirmed by semi-quantitative RT-PCR using both rat and human cultured cells. Consistent with these results, hepatocyte growth factor preferentially stimulated the growth of rat FP cells, whereas PDGF-AA preferentially stimulated rat fibroblasts. High concentrations of some these kinases are also found in the follicular matrix keratinocytes as revealed by *in situ* hybridization. The expression of specific tyrosine kinases in FP and matrix cells may play roles in regulating hair growth and cycling.

Key words: hair follicle/dermal papilla/PDGF/HGF/c-Met
J Invest Dermatol 123:283–290, 2004

The hair follicle is a dynamic structure that undergoes cyclic growth (Muller-Rover *et al*, 2001). Although several growth factors and hormones have been implicated in regulating hair growth, relatively little is known about the genes that are involved in follicular regulation. It is well established that follicular papilla (FP), a group of specialized mesenchymal cells, is able to support hair growth, whereas their embryologically closely related dermal fibroblasts (F) cannot do so (Oliver, 1967; Ibrahim and Wright, 1982; Jahoda *et al*, 1984; Kamimura *et al*, 1997; Jahoda *et al*, 2001). We have therefore been interested in the identification of genes that are preferentially expressed in FP cells (Yu *et al*, 1995, 2001), and to study their possible roles in hair regulation.

Tyrosine kinases are known to play important roles in cell growth and differentiation (Weiss *et al*, 1997; Weiss and Schlessinger, 1998; Hsueh and Scheuermann, 2000; Rebay, 2002). More than half of the known tyrosine kinases are growth factor receptors, whereas the others are non-receptor components of signaling transduction pathways, products of oncogenes of acutely transforming retroviruses, etc. The receptor kinases consist of three distinct domains: an extracellular domain that binds the growth factor ligand, a transmembrane domain, and an intracellular domain consisting of the catalytic domain of tyrosine kinase (Ullrich and Schlessinger, 1990). Receptor activation results from ligand binding to two neighboring receptors or subunits, thus inducing conformational changes of the receptors. This signal is transmitted to the intracellular domain of the receptor complex, initiates a cascade of intracellular reactions, and eventually results in cellular responses, such as cell proliferation and migration. Usually, these intracellular signal transduction events are regulated by some other tyrosine kinases. After being phosphorylated, some cytoplasmic proteins are activated and react with their downstream targets either in the cytoplasm or in the nucleus (Lemmon and Schlessinger, 1994).

Since tyrosine kinases are known to play key roles in cell proliferation and differentiation in many tissues, it would be interesting to see (i) whether FP cells preferentially express some particular tyrosine kinases *versus* dermal fibroblasts (F); and (ii) what possible functions these FP-specific tyrosine kinases have in regulating hair growth. To begin to study these issues, we compared the tyrosine kinase expression profiles of cultured rat vibrissa FP cells and skin fibroblasts both at passage 3–4 and 5-d post-confluence. We decided to compare these two cell types grown in culture because (i) there is evidence that early passage cultured FP cells, but not cultured dermal fibroblasts, retain the capacity to support hair growth (Jahoda *et al*, 1984); (ii) one can obtain large amounts of cells thus facilitating their analysis, (iii) we have shown that the passage 3–4 cultured rat FP cells can support *in vivo* hair reconstitution using the system described by Lichti *et al* (1995); (iv) these two cell types are both in a nearly quiescent state *in vivo* thus we used 5-d post-confluent cells instead of replicating cultures; and (v) our previous studies indicate that the comparison of FP and F cells that have been grown under identical tissue culture conditions can lead to the identification of dermal

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; F, dermal fibroblast; FP, follicular papilla; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor

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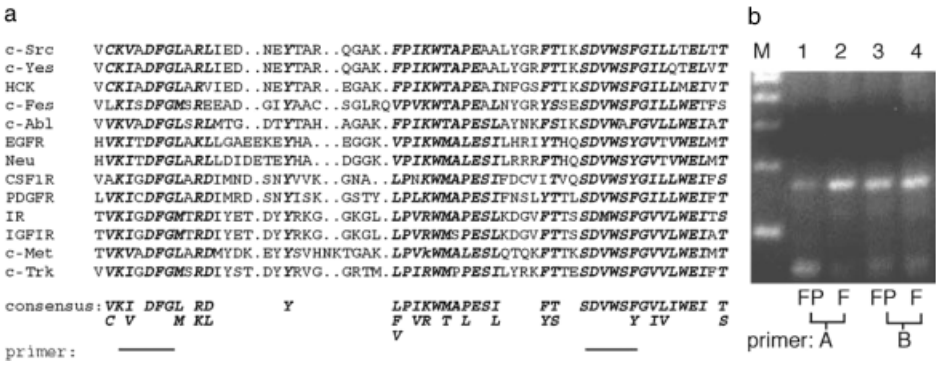

Figure 1
Amplification of tyrosine kinase by RT-PCR. (*a*) Amino acid sequence alignment of the catalytic domains from various tyrosine kinases (Wilks, 1989). Dots correspond to gaps introduced to maximize sequence alignment (each dot represents one amino acid). Conserved regions are shown in bold italic type and the derived consensus sequences are shown below. The two horizontal lines mark the two motifs (DFG and DVW) used for designing the degenerate PCR primers. (*b*) PCR was performed using cDNA of cultured rat vibrissa follicular papilla cells (FP, *lanes 1* and *3*) and dermal fibroblasts (F, *lanes 2* and *4*) as templates. To adequately cover all the possible amino acid sequences within these two conserved motifs and to avoid excessive primer degeneracy, we synthesized two separate sets of primers: pair A (*lanes 1* and *2*) and pair B (*lanes 3* and *4*). The PCR products, together with a 100-bp DNA molecular size maker (M), were separated in a 4% agarose gel and stained with ethidium bromide. Note that DNA fragments of $\sim$ 170 bp were amplified in all PCR reactions.

papilla-specific genes including nexin-1 (Yu *et al*, 1995) and osteopontin (Yu *et al*, 2001).

In this paper, we compared the tyrosine kinase profile of FP and F cells that had been cultured under identical conditions. Since all tyrosine kinase family members share a highly conserved catalytic domain, we utilized this feature to design degenerate PCR primers (Fig 1) (Wilks, 1989; Robinson *et al*, 1996). Analysis of the tyrosine kinase profiles using differential display as well as random cloning showed that cdc2, c-met, tec, tyro 10, and sky kinases were preferentially expressed in FP cells, whereas α-platelet-derived growth factor receptor (α-PDGFR) was preferentially expressed in fibroblasts. We confirmed the cell-type specificity of some of these tyrosine kinases by semi-quantitative RT-PCR, and by functional cell growth assays. Finally, we showed that matrix keratinocytes also express high levels of some of these kinases, at a level much higher than that of other skin cell types.

Results

Tyrosine kinase profiles of cultured rat FP cells and dermal fibroblasts To identify the tyrosine kinases that were preferentially expressed in FP cells, we used two independent approaches to analyze the degenerate PCR products, i.e., differential display and random cloning. In the first approach, we digested the $\sim$ 170-bp PCR products (Fig 1) using a number of restriction enzymes and separated electrophoretically the fragments on a 6% polyacrylamide gel. We found that whereas the majority of the DNA fragments were identical in FP cells and fibroblasts, consistent with their close embryological relationship, several fragments were present at much higher levels in one cell type than the other (Fig 2). These differentially expressed tyrosine kinase fragments were cloned and identified. Using this approach, we found that cdc2, c-met, tec, tyro 10, and sky were preferentially expressed in cultured rat FP cells, whereas α-PDGFR was preferentially expressed in fibroblasts (Table I).

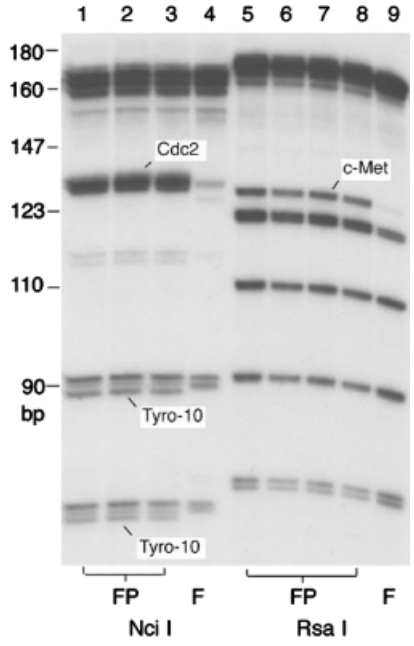

Figure 2
Differential display of the restriction fragments of various tyrosine kinases. The $\sim$ 170-bp PCR products of tyrosine kinases, as amplified from cultured rat vibrissa follicular papilla cells (FP; *lanes 1–3* and *5–8*) and dermal fibroblasts (F; *lanes 4* and *9*), were digested with various restriction enzymes, i.e., Nci I (*lanes 1–4*) and Rsa I (*lanes 5–9*), and separated on a 6% polyacrylamide gel. Numbers on the left denote the positions of size markers in base pair (bp). Note the detection of several FP-specific cDNA fragments of cdc2, tyro-10, and c-Met (as marked).

We also analyzed the tyrosine kinase profiles by random cloning and sequencing of the 170-bp bands of the two cell types. We digested the 170-bp PCR products of the two cell types using BstEII, and cloned the released DNA into a modified BlueScript vector. Thirty-five clones were randomly picked from the PCR products generated with pair A or B primers and sequenced. Using this approach, we found that cdc2, c-met, and tec were preferentially expressed in FP cells, whereas α-PDGFR and c-abl were preferentially expressed in fibroblasts. Another six tyrosine

Table I. Tyrosine kinases identified by differential display

Cell	Kinase	Restriction enzyme(s)
DP > F	Cdc2	Ava II, BaJ I, BssK I, Hae III, MnL I, Msp I, Mwo I, Nci I, NLa IV, Rsa I, Sau96 I
	c-Met	Ava II, Hinf I, Msp I, NLa III, Rsa I, Tai I
	Tec	Aci I, EcoR II, Fnu 4H1, Hae III, Mwo I
	Tyro 10	Aci I, EcoR II, Hae III, Msp I, Nci I, NLa IV, Tsp45 I
	Sky	Nci I
DP < F	α-PDGFR	C8C8 I, Dde I, Hinf I, Mse I, NLa III

α-PDGFR, α-platelet-derived growth factor receptor.

Table II. Tyrosine kinases identified by random cloning

Cell	Kinase	Clone # (primer A + B)	
		DP	F
DP > F	Tyro10	19 (11 + 8)	11 (4 + 7)
	Cdc2	10 (0 + 10)	1 (0 + 1)
	c-Met	5 (5 + 0)	0 (0 + 0)
	Tec	4 (3 + 1)	1 (0 + 1)
DP < F	α-PDGFR	0 (0 + 0)	16 (16 + 0)
	c-Abl	4 (3 + 1)	11 (2 + 9)
DP = F	Ufo	6 (1 + 5)	7 (3 + 4)
	IGFR	6 (6 + 0)	5 (5 + 0)
	MARK2	3 (0 + 3)	4 (0 + 4)
	Fyn	3 (2 + 1)	3 (1 + 2)
	β-PDGFR	3 (3 + 0)	2 (2 + 0)
DP ? F	Mpk3	2 (1 + 1)	0 (0 + 0)
	FGFR	1 (0 + 1)	0 (0 + 0)
	Sky	1 (0 + 1)	0 (0 + 0)
	SMT-S/T-K	1 (0 + 1)	0 (0 + 0)
	PTK	1 (0 + 1)	0 (0 + 0)
	Neu	0 (0 + 0)	2 (0 + 2)
	18s, 5.8srRNA	0 (0 + 0)	2 (0 + 2)
	S/T kinase	0 (0 + 0)	1 (1 + 0)
	CHED	0 (0 + 0)	1 (0 + 1)
	Sty	0 (0 + 0)	1 (0 + 1)
	Ca^{2+} PK	1 (0 + 1)	1 (1 + 0)
Total		70 (35 + 35)	70 (35 + 35)

α-PDGFR, α-platelet-derived growth factor receptor. IGFR, insulin growth factor receptor; FGFR, fibroblast growth factor receptor.

kinases, i.e., tyro 10, ufo, IGF receptor, MARK2, fyn, and β-PDGFR, were found about equally in the two cell types (Table II). These results were consistent with the data obtained from the differential display approach, and confirmed that cdc2, c-met, and Tec were overexpressed in FP cells.

Consistent with the finding that cdc2 is overexpressed in cultured FP cells, we found that several molecules that are involved in regulating cdc2 activity, including chk1, cyclin B, and wee1, were also overexpressed in cultured follicular cells (Fig 3; *lanes 4, 6*, and *8*) in comparison with cultured fibroblasts (Fig 3; *lanes 5, 7*, and *9*).

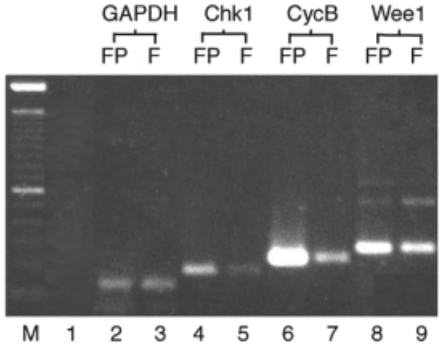

Figure 3
Comparison of the cdNAs encoding several proteins involved in regulating the cdc2 activities. Chk1, cyclin B and wee-1 cDNA's were amplified using as templates the cDNA's of rat FP cells (*lanes 2, 4, 6*, and *8*) and fibroblasts (*lanes 3, 5, 7*, and *9*). *Lane 1* denotes a negative control. GAPDH was used to normalize the amount of cDNA. Note the higher amounts of chk1 and cyclin B cDNAs in dermal papilla cells.

Confirmation of FP-specific tyrosine kinases To confirm that the tyrosine kinases identified above were cell-type specific (or "enriched"), we performed semi-quantitative RT-PCR to compare the cDNA levels of these tyrosine kinases in cultured rat and human FP *versus* fibroblasts. GAPDH was analyzed to normalize the amount of the cDNA templates. We found that the expression levels of cdc2, c-met, and tec were indeed higher in rat FP cells than those in rat fibroblasts (Fig 4*a*, *c*, and *e*). Consistent results were obtained in cultured human cells (Fig 4*b*, *d*, and *f*); the only exception was that α-PDGFR was almost equally expressed in human FP cells and fibroblasts possibly related to the site variation of the cells (Fig 4*h*; cf. rat data in Fig 4*g*). Overall, these results confirmed the cell-type specificity of most of the tyrosine kinases as identified by differential display and random cloning.

FP-specific tyrosine kinase growth factor receptors - Since some of the tyrosine kinases that we identified were growth factor receptors, one would predict that these two cell types should respond differently to the corresponding growth factors. Indeed, consistent with the overexpression of c-met (HGF receptor) in FP cells (Tables I and II, Figs 2

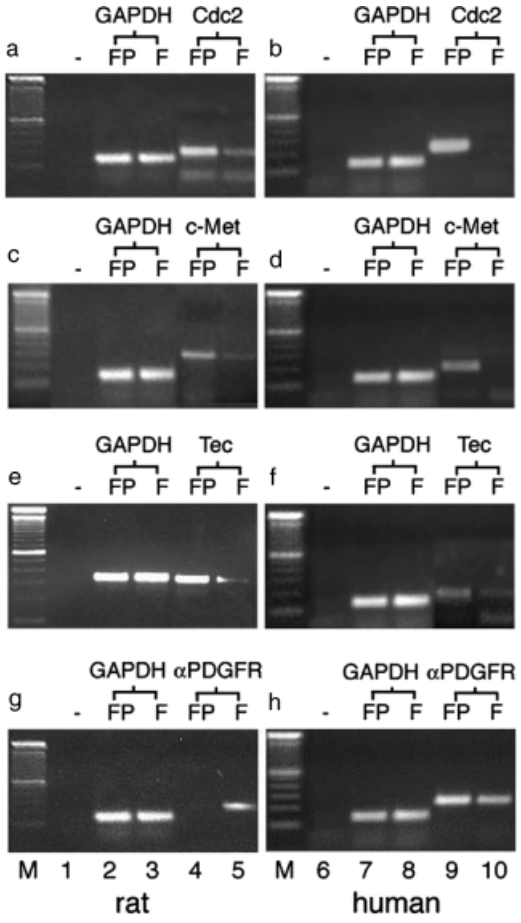

Figure 4
Confirmation of the cell-type specificity of the tyrosine kinases by semi-quantitative PCR. PCR was performed on cDNA templates of cultured rat vibrissa follicular papilla (FP) cells (*lanes 2* and *4*), rat dermal fibroblasts (*lanes 3* and *5*), human scalp FP cells (*lanes 7* and *9*), and human scalp fibroblasts (*lanes 8* and *10*). (*a* and *b*) cdc 2; (*c* and *d*) c-met; (*e* and *f*) tec; (*g* and *h*) α-platelet-derived growth factor (α-PDGF) receptor. GAPDH (*lanes 2, 3, 7*, and *8*) was used to normalize the amounts of cDNA templates from FP and F cells. *Lanes 1* and *6* were negative controls using distilled water as the template. M denotes 100-bp DNA molecular size markers. Note the higher amounts of cdc2 (*a* and *b*), c-met (*c* and *d*), and tec (*e* and *f*) in both rat and human FP cells, and the higher amount of α-PDGF receptor in rat fibroblasts (*g*).

and 4), we found that HGF stimulated the proliferation of rat FP cells in a concentration-dependent manner (maximal effect at 50 ng per mL), but with minimal effects on cultured fibroblasts (Fig 4*a*). Similarly, we found that PDGF AA stimulated the growth of cultured rat fibroblasts, but not FP cells, consistent with the overexpression of α-PDGF receptor in the former cells (Fig 5*b*). We also found that PDGF BB (which binds to both α- and β-PDGF receptor) indeed stimulated the growth of both human follicular and dermal fibroblasts (Fig 5*c*), again consistent with the random cloning data showing that β-PDGF receptor was expressed at similar levels in the two human cell types (Table II).

Expression of kinases in matrix keratinocytes *In situ* hybridization studies showed that c-met, cdc2, and Tec were expressed intensely in the epithelial matrix adjacent to the FP whereas, in comparison, only weak signals were detected in the papilla. The tremendous high level of signals in the matrix made it difficult to access the level of expression in FP (Fig 6; see Discussion). Similar results were obtained with vibrissa and back skin follicles (data not shown). The intense signals of c-met, cdc2, and tec in the matrix, interestingly, seemed to be associated with the lower or regenerative matrix, and upper matrix, respectively (Fig 6).

Discussion

We studied the tyrosine kinase profiles using both differential display and random cloning. These screening methods are not exhaustive. If we were to use more restriction enzymes in the differential display, or to sequence more clones in random cloning, we probably would have identified additional kinases. Based on the Human Genome Program Unigene Resources, an experimental system for automatically partitioning Genebank sequences into a non-redundant set of gene-oriented clusters, there are a total of $\sim$ 920 known human kinases. About 10% of them, are tyrosine kinases and, of these, over half are receptors that can be further divided into 20 subfamilies (Robinson *et al*, 2000). Mouse orthologs can be identified for almost all these human tyrosine kinases, indicating a high degree of conservation. Although the exact number of tyrosine kinases in a given cell type is unknown, we have identified 22 tyrosine kinases in cultured rat vibrissa and dermal fibroblastic cells (Tables I and II). Although our lists are almost certainly incomplete, the

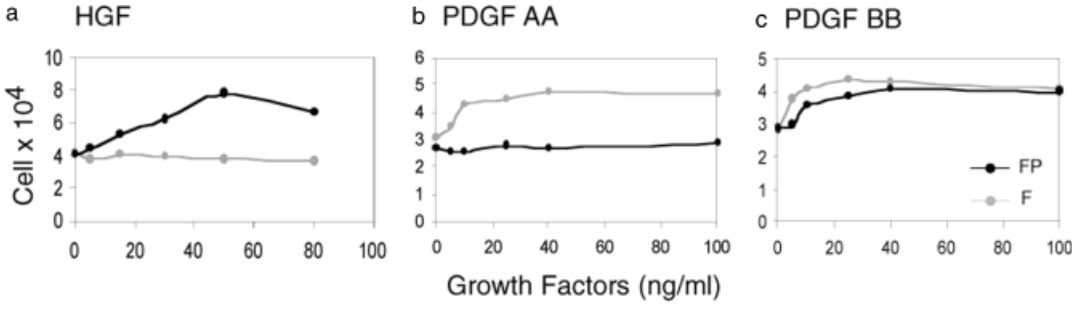

Figure 5
Differential responses of cultured follicular papilla (FP) cells and fibroblasts to growth factors. Rat vibrissa FP cells and fibroblasts (1–2 $\times$ 10^4 cells) were plated in 35-mm dish and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) for 2 d, and fed with DMEM containing 3% FBS with or without hepatocyte growth factor (HGF) (*a*), α-platelet-derived growth factor (PDGF) AA (*b*), or PDGF BB (*c*). Cell number was counted 72 h later. Note that, as predicted by the tyrosine kinase profiles, HGF preferentially stimulated the growth of rat FP cells (*a*), PDGF AA preferentially stimulated the growth of fibroblasts (*b*), and PDGF BB stimulated the growth of both cell types (*c*).

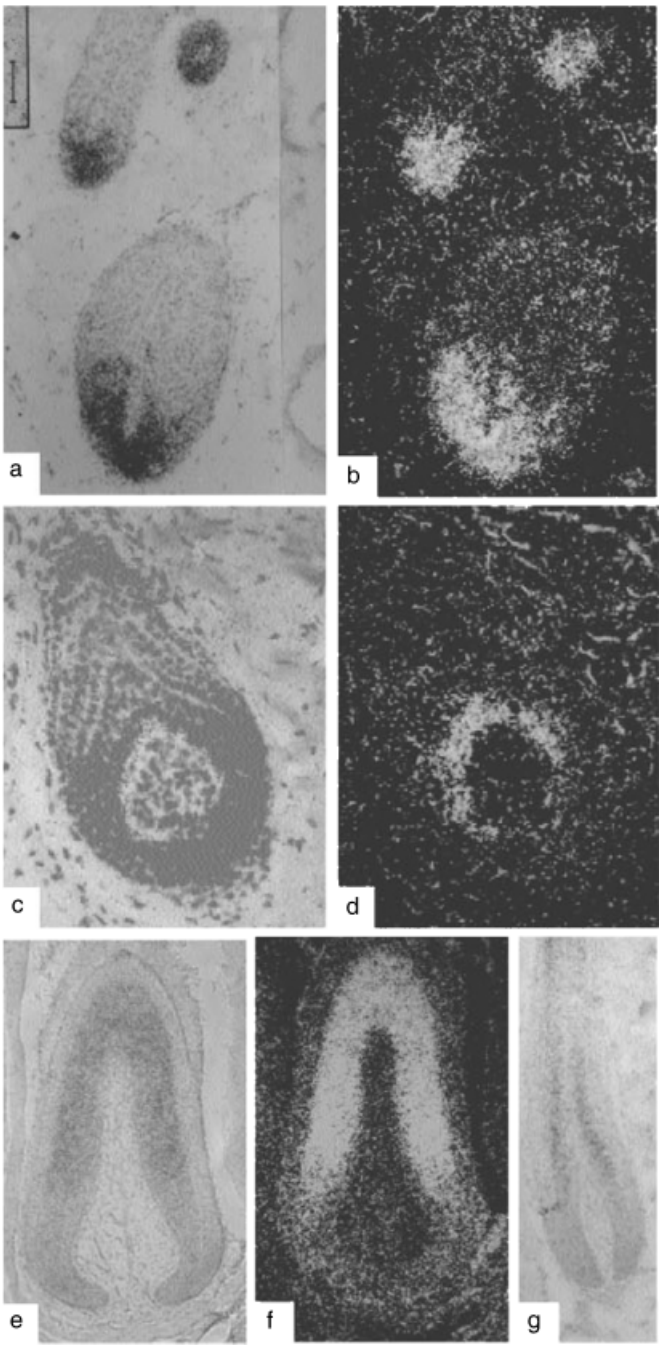

Figure 6
***In situ* localization of the mRNA's of c-Met (*a*, *b*), cdc2 (*c*, *d*), and tec (*e–g*).** Panels (*a, c, e* and *g*) and (*b, d*, and *f*) are light and dark fields, respectively. Panels *a*, *b*, *e*, and *f* are rat vibrissa follicles, whereas panels *c*, *d* and *g* show rat back skin follicles. Note the heavy silver grains in the matrix portion of anagen follicles making it difficult to assess precisely the silver grain levels in the adjacent follicular papilla.

remarkable consistency between our random cloning and restriction identification data suggest that we have identified most of the major tyrosine kinases that are present in cultured rat vibrissa FP cells and dermal fibroblasts.

Using differential display and random cloning, we have shown that cdc2, c-met, tec, and tyro 10 are preferentially expressed in cultured rat vibrissa FP cells, whereas α-PDGF receptor is preferentially expressed in cultured rat dermal fibroblasts. Of all these genes, only c-met (HGF receptor) and PDGF receptor have been reported to be involved in hair growth and development.

HGF is usually synthesized by mesenchymal cells and targets neighboring epithelium or endothelium (Boros and Miller, 1995; Birchmeier *et al*, 1997; Jiang and Hiscox, 1997; Birchmeier and Gherardi, 1998). In the hair follicle, it is known that (i) HGF is secreted by FP (Shimaoka *et al*, 1995); (ii) HGF can stimulate the proliferation of cultured human bulb keratinocytes (Shimaoka *et al*, 1995); (iii) HGF can promote the elongation of mouse vibrissa and human follicle in a concentration-dependent manner in organ culture (Jindo *et al*, 1994); and (iv) HGF and its c-Met receptor appear to be expressed in the FP and hair bulb epithelial cells, respectively, and this HGF/met signaling may play a role in protecting the follicular epithelial cells from apoptosis that occurs in catagen (Lindner *et al*, 2000). These data suggest that HGF serves as a paracrine factor in the hair follicle, passing signals from mesenchyme to epithelium; however, it has been shown recently that HGF can also function as an autocrine factor in certain tissues (Andermarcher *et al*, 1996; Anastasi *et al*, 1997). For example, HGF and its receptor c-Met have been reported to co-express in myoblasts forming an autocrine loop (Anastasi *et al*, 1997). Our result showing that cultured FP cells expressed high levels of c-Met (Figs 2 and 4) suggests that an autocrine HGF loop can, under certain conditions, operate in the FP. Furthermore, we showed that HGF can stimulate the proliferation of cultured rat vibrissa FP cells (Fig 4), thus proving that the Met receptor of the FP cells can indeed respond to its HGF ligand. These data suggest that HGF may act both as a paracrine and (potentially) an autocrine factor in follicular regulation.

PDGF-A and its receptor, α-PDGF receptor, are known to be involved in hair follicle development. Both PDGF-A and α-PDGFR knockout mice show severe defects in embryos and die shortly after birth. PDGF-A knockout mice develop smaller FP, thinner hair, misshapen follicles, and thinner dermis (Karlsson *et al*, 1999). PDGFR-α knockout mice have regional detachment of the epidermis (blebs) in early embryo due to a loss of the dermal mesenchyme (Schatteman *et al*, 1992). Akiyama *et al* (1996) reported that α-PDGFR is detected in the follicular sheath of fetal human hair follicle but not in the FP. Orr-Urtreger showed that the expression of PDGF-A in epidermis and of α-PDGFR in the underlying mesenchyme begins before the onset of pelage hair morphogenesis (Orr-Urtreger and Lonai, 1992). Our data showed that cultured adult rat vibrissa FP cells do not synthesize α-PDGF receptor, and PDGF AA has no effect on the growth of these cultured cells (Fig 4). If α-PDGF receptor is indeed absent in FP *in vivo*, perhaps α-PDGF receptor is involved in hair morphogenesis only in an early developmental stage. Another possibility is that α-PDGF receptor is absent only in vibrissa FP, but is present in the follicular papillae of pelage hairs.

The formation of cdc2/cyclin B complex and the nuclear translocation of this complex initiate the M-phase of mitosis (Kishimoto and Okumura, 1997). Our finding that cultured rat FP cells overexpress cdc2 kinase, *versus* cultured dermal fibroblasts, was unexpected, since in general cultured follicular cells proliferate slower than fibroblasts and since both cells were harvested at 5-d post-confluence

when cell division was minimal. Interestingly, the higher cdc2 message level in follicular cells, although accompanied by high message levels of cyclin B, may be balanced by the overexpression of chk1—a protein kinase that can potentially inactivate cdc25 thus resulting in the suppression of the cdc2 activity. Although the physiological meaning of the overexpression of cdc2 and some of its regulatory proteins in FP cells is unclear, it will be interesting to study the possible role of these cell cycle proteins in apoptosis given the strong link between cell cycle checkpoints and apoptosis (King and Cidlowski, 1995; Pandey and Wang, 1995).

Tec, a non-receptor tyrosine kinase, belongs to a family of non-receptor, cytoplasmic tyrosine kinases. It is highly expressed in many hematopoietic cell lines, and is implicated in antigen receptor signaling required for the survival and differentiation fate of the cell, including the proper lymphocyte activation and development (Sato *et al*, 1994; Lewis *et al*, 2001). Tec family kinases are activated following ligand stimulation of a number of cell surface molecules including receptor tyrosine kinases, antigen receptors, cytokine receptors, and G-protein-coupled receptors (Schaeffer and Schwartzberg, 2000). The activated Tec-related kinases form a critical intermediate complex in lymphocytes leading to PLC-gamma phosphorylation, calcium mobilization, and activation of protein kinase C. Many proteins are known to interact with the Tec family kinases including Src family kinases, c-Kit, various SH-domain adaptors, and a number of novel Tec-interacting proteins (Schaeffer and Schwartzberg, 2000). In this regard, it would be interesting to see whether FP cells contain any tissue-restricted Tec-binding proteins and whether they can be a target for hair modulation.

Tyro 10 is a receptor tyrosine kinase, which is widely expressed in brain, heart, lung, and some other tissues, whereas its extracellular (ligand binding) domain is homologous to cell surface mediators of protein–protein interactions, including blood coagulation Factors V and VIII, and the neuronal recognition protein A5 (Karn *et al*, 1993). Its catalytic domain exhibits significant similarity to the neurotrophin receptor, another tyrosine kinase family member (Lai and Lemke, 1994). The biological role(s) and even the ligand of tyro 10 are still unknown.

We could not meaningfully analyze the expression of the Met, cdc2, and Tec kinases in the FP *in vivo* because of these kinases are expressed at much higher levels in the adjacent matrix keratinocytes (Fig 6). The "spillover" of the matrix signals into the papilla area that has a much lower level of expression made it technically difficult to access the true level in the papilla. We believe that our data on cultured FP and DF cells may reflect a particular *in vivo* state of these cells such as certain stages of skin development or regeneration. As far as the kinase expression pattern in the matrix is concerned, the c-met message seems to be associated with the lowest or germinative matrix keratinocytes, the mRNA of cdc2 is present in tremendous amounts in the lower matrix corresponding to the cell division zone, whereas the tec kinase is expressed in an extremely high level in the upper matrix (above the cdc2 zone). Such a differential distribution of the three kinases is interesting. For example, the expression of tec in upper matrix suggests that it may be involved in the transcription of some hair follicle-specific, differentiation-dependent genes, such as those encoding hair keratins. The expression levels of some of these kinases in the matrix are so high that one can hardly see the signals in any other cells in the skin including the epidermis, fibroblasts, muscle cells, and melanocytes. The fact that these kinases are practically hair follicle-"specific" raises the interesting possibility that drugs that can selectively inhibit, e.g., Tec, may specifically interfere with follicular differentiation and thus provide a way for hair removal. Interfering the expression of other genes that are expressed in large amounts in follicular cells, e.g., nexin-1 or osteopontin (Yu *et al*, 1995, 2001), could potentially have similar hair removal effects.

Materials and Methods

Cell culture and growth factor treatment All animal studies have been approved by institutional IRB. Vibrissa follicles were isolated individually from the lip region of 4–6-mo-old male Wistar rats (Charles River). To expose the FP, the lower part of the follicle was opened by a 20-gauge needle. About 35–40 FPe can be microdissected from each rat. The isolated FPe were placed in 1 mL Chang's medium (Irvine Scientific, Santa Ana, California) with 100 U per mL penicillin and 100 μg per mL streptomycin in a 35-mm Petri dish, and left undisturbed in an incubator (at 37°C and 5% CO_2) for 4 d (Jahoda and Oliver, 1981, 1984; Warren *et al*, 1992); the culture medium was changed every 3 d thereafter. Ten to 12 d later, the explant cultures were treated with 0.125% trypsin and 0.01% EDTA in phosphate-buffered saline (PBS), and the dissociated single cells were then plated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). These FP cells were subcultured at $\sim$1:3 ratio and were used at passage 3–4. Rat dermal fibroblasts were similarly cultured by explant outgrowth of small pieces (<1 mm^3) of interfollicular dermis from the same lip skin tissue, from which the vibrissa follicles had been removed. Human FP cells were isolated from occipital scalp and cultured as described above.

In the growth factor treatment experiments, subcultured FP cells and fibroblasts (1–2 $\times$ 10^4 cells) were plated in 35-mm dishes and cultured in DMEM containing 10% FBS for 2 d. After washing with PBS, the cells were fed with DMEM containing 3% FBS plus HGF, PDGF AA or PDGF BB (R & D Systems, Minneapolis, Minnesota). The series of concentrations used to treat the cells were as follows: HGF: 5, 15, 30, 50, and 80 ng per mL; PDGF AA and PDGF BB: 5, 10, 25, 40, and 100 ng per mL. Cell number was counted 72 h after the addition of the growth factors.

cDNA synthesis and PCR using degenerate primers Total RNA was isolated from FP and F cells at 5-d post-confluency with a kit (RNAgents Total RNA Isolation System, Promega, Madison, Wisconsin) using guanidine thiocyanate and citrate/sarcosine/β-mercaptoethanol as the denaturing buffer, followed by phenol extraction. Twenty micrograms of total RNA were reverse transcribed to first-strand cDNAs with oligo $(dT)_{12–18}$ (Gibco, Carlsbad, California).

We compared the tyrosine kinase profiles of cultured FP cells *versus* fibroblasts. Degenerate primers were used to amplify a region of the kinases flanked by DFG and DVW motifs (as defined in Fig 1). This region consists of the catalytic domain and is located close to the 3′-end of the tyrosine kinase transcripts (Fig 1*a*) (Wilks, 1989; Robinson *et al*, 1996; Chen *et al*, 1999). Between these two primer sequences, there is another highly conserved region, P(V/I)(K/R)W(M/T)APES, which can be used to confirm the tyrosine kinase nature of the transcripts. In the mean time, the sequences between the two primers are diverse enough to distinguish individual family members. Moreover, the relatively constant size of the PCR products (about 170 bp), which reflects the relatively

fixed number of nucleotides between the conserved DFG and DVW motifs, greatly simplifies the purification and characterization steps. To increase the chance of digestion, we used several restriction enzymes recognizing 4-bp motifs to perform the differential display. Theoretically, these enzymes should have a cutting site, in average, in every 256 bp; restriction enzymes with longer recognition sites will yield too few cuts to be practical.

The PCR primers were designed based on the conserved DFG and DVW motifs of tyrosine kinases, with 5′-BstEII adaptor (underlined in the following primer sequences) (Fig 1) (Wilks, 1989; Robinson *et al*, 1996; Chen *et al*, 1999). Three to four additional nucleotides were added at the 5′ to the BstEII adaptor, in order to facilitate the restriction enzyme digestion. To adequately cover all the possible amino acid sequences within these two conserved motifs and to avoid excessive primer degeneracy, we synthesized two separate sets of primers, A and B. The nucleotide sequences of the primers were as follows (5′ → 3′):

5′-primer A: CCAGGTCACCAAA(G)A(G)TIT(A/G)GIGATTTTGG
B: CCAGGTCACCAAA(G)A(G)TIT(A/G)CIGATTTTGG
3′-primer A: CACAGGTTACCA(G)A(T)AIGA(C)CCAIACA(G)TC
B: CACAGGTTACCA(G)A(T)AA(G)CTCCAIACA(G)TC

Two annealing temperatures were used for the degenerate PCR: 42°C for five cycles and 55°C for additional 25 cycles. The PCR products were analyzed on a 4% agarose gel.

Restriction enzyme digestion and differential display The degenerate PCR products were treated with various restriction enzymes, including Aci I, Ava II, BaJ I, BssK I, C8C8 I, Dde I, EcoR II, Fnu 4H1, Hae III, Hinf I, MnL I, Mse I, Msp I, Mwo I, Nci I, NLa III, NLa IV, Rsa I, Sau96 I, Tai I, and Tsp45 I. The digested DNA fragments were resolved electrophoretically on a 6% polyacrylamide gel. The area of a dried gel containing a DNA fragment of interest was excised, and the DNA was extracted by heating the gel slices in distilled water at 90°C for 5 min. The solubilized DNA was converted to blunt-end fragment using T4 DNA polymerase and dephosphorylated using calf intestine phosphatase (CIP) (37°C for 1 h) and ligated into PT-blunt vector by T4 DNA ligase overnight at room temperature. After transformation and amplification in bacteria, individual clones were picked and sequenced.

Random cloning The PCR products amplified using the degenerate primers as described above were electrophoresed on a 4% agarose gel. The ~ 170-bp band was purified from the agarose (Qiagen, Valencia, California) and digested with BstEII restriction enzyme. A modified pBlueScript (+/−) vector, which was inserted with an EcoRI–BstEII–HindIII adaptor, was also digested by BstEII. The BstEII-digested PCR products and vector were ligated overnight at 14°C. After transformation and amplification, individual plasmid clones were picked randomly and their DNA was sequenced according the dideoxy method (Sanger *et al*, 1977) using T7 Sequenase DNA polymerase (Amersham Life Science, Piscataway, New Jersey) and [^{35}S]dATP (Dupont/New Life Science, Boston, Massachusetts).

Semi-quantitative RT-PCR Five micrograms of total RNA from cultured FP cells and dermal fibroblasts were reverse transcribed into cDNA (Gibco). The 1:1000 diluted cDNA was used as PCR template and normalized against the GAPDH level. The thermal cycling program was set up as following: one cycle at 95°C, 4 min; 30–35 cycles at 95°C, 45 s; 55°C, 45 s; 72°C, 2 min; and one cycle at 72°C, 10 min. The sequences of forward and reverse primers of each gene were as following (forward and reverse; both listed 5′ → 3′): cdc2 (GTCCCACGTCAAGAACCTGG, AGGAAAAGTCCAGATGATGG); c-met (GTTCACCACCAAGTCAGACG, CGCCGTCAATGTTGTCTTGG); Tec (CAGTACACAAGTTCTTCTGG, ATCTCAGCATCACCTCATAC); α-PDGFR (AGAGGAACAGACACAGCTCG, GCCAGTTAAAGGAAGCTGTC); and GAPDH (ATCACCATCTTCCAGGAGCG, GGGTGGCAGTGATGGCATGG).

***In situ* hybridization** The PCR products of cdc2, c-met, and Tec were subcloned into pBluescript, and the sense and antisense RNA transcripts were generated in the presence of ^{35}S-UTP using T3 or T7 enzymes. *In situ* hybridization was conducted as described (Lavker *et al*, 1988).

This work was supported by a grant from Anaderm/Pfizer. We thank Dr Hsing-Jien Kung (University of California, Davis) and Robert M. Lavker (Northwestern University Medical School) for helpful suggestions.

DOI: 10.1111/j.0022-202X.2004.23212.x

Manuscript received March 5, 2003; revised March 8, 2004; accepted for publication March 9, 2004

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